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ANALYSIS OF THE MYOSTATIN GENE (*MSTN*) POLYMORPHISM IN FOUR BREEDS OF HORSES

ANALIZA POLIMORFIZMU GENU MIOSTATYNY (*MSTN*) U CZTERECH RAS KONI

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Streszczenie. Gen kodujący miostatynę (*MSTN*) jest markerem optymalnego dystansu biegu, wskaźników szybkości oraz budowy ciała koni wyścigowych czystej krwi. Ponieważ wykazuje on dużą zmienność w zależności od rasy oraz typu użytkowego, zaprojektowano test do badania polimorfizmu g.66493737C/T w 1 intronie genu *MSTN* u koni, oparty na metodzie PCR; przeprowadzono analizę występowania wariantów g.66493737C/T u czterech ras koni hodowanych w Polsce. Badania przeprowadzono na 209 koniach reprezentujących rasy: holsztyńską, polski koń szlachetny półkrwi, polski koń zimnokrwisty i konik polski. Do analizy genu *MSTN* zastosowano metodę ACRS (ang. *amplification created restriction site*). Trawienie amplikonów genu *MSTN* (132pz) enzymem *Rsa*I pozwoliło na identyfikację poszczególnych genotypów na podstawie długości fragmentów restrykcyjnych: *TT* – 132pz, *CT* – 132, 103, 29pz i *CC* – 103, 29pz. Na podstawie wyników stwierdzono, że genotyp *CC* (typ szybki, sprint) był obecny tylko u koni rasy holsztyńskiej (0,073), której przodkami były konie czystej krwi angielskiej. Genotyp *CT* (typ szybki, średni dystans) ujawnił się z najwyższą frekwencją u polskich koni szlachetnych półkrwi (0,735). Obie rasy reprezentują typ wierzchowy. Ostatni genotyp – *TT* (większa wytrzymałość) był najczęstszy u polskiego konia zimnokrwistego (0,830) i konika polskiego (0,736), które zalicza się odpowiednio do typu ciężko-zaprzęgowego i wszechstronnie użytkowego. Różnice frekwencji genotypów pomiędzy rasami były statystycznie istotne ($p \leq 0,05$ dla *CC*, $p \leq 0,01$ dla *TT* i $p \leq 0,001$ dla *CT*). Wyniki niniejszych badan wykazały istotne zróżnicowanie wariantów genu *MSTN* w zależności od typu użytkowego i rasy koni hodowanych w Polsce.

Key words: ACRS, horses, *MSTN,* polymorphism.
Słowa kluczowe: ACRS, gen *MSTN*, konie, polimorfizm.

INTRODUCTION

All the horses, independent of utility type serve the people by their work, so it is very important to recognize genetic background of physical performance in these animals. Genes which determine physical performance may be responsible for structure and power of muscles, their interactions, metabolic pathways, oxygen utilization, lactate metabolism and fatty acids oxidations.

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One of the key gene influencing horse musculature is gene encoding myostatin – *MSTN*. Myostatin is a negative regulator of skeletal muscles growth which is responsible for their proper development. There are many *MSTN* gene variants which are strongly associated with muscle hypertrophy phenotypes, mainly in cattle, dogs, mice as well as human (Grobet et al. 1997; McPherron et al. 1997; Schuelke et al. 2004; Mosher et al. 2007). Equine *MSTN* gene was characterized for the first time at the end of last century (Caetano et al. 1999). It is localized on chromosome 18. (Eca18) and consists of three coding exons. The length of transcript is 1128bp, however protein is composed of 374aa (Ensembl). Investigations of equine *MSTN* gene were continued by one decade till their variants, which could be applied as a markers for speed or endurance in horses were determined. One of them is described as "speed gene" and is localized in genomic position g.66493737. Its different variants allow to predict best race distance with high probability in Thoroughbreds. *CC* genotype is suitable for fast speeds at short-distance races, *CT* for middle-distance races, however *TT* genotype for greater stamina in horses running at the longer distance races (Hill et al. 2010). Tozaki et al (2010) also indicated that *C* allele is desirable for winning and surviving Thoroughbreds at the early stages of their athlete life in Japan Racing Association racing programs.

The aim of this study was to design PCR based test for g.66493737C/T polymorphism detection and to analyze its frequency in horses raised in Poland which belong to different breeds and utility types.

MATERIAL AND METHODS

Investigations were carried out on the sample of horses consists of:

- warm-blooded half-bred horses, saddle type – Holstein Horse (n = 69) and Polish Noble Half-breed (n = 34);
- cold-blooded horses, heavy-draught type – Polish Heavy Draft (n = 53);
- primitive horses, general utility type – Polish Konik (n = 53).

DNA was isolated from blood by use MasterPure^{TM} DNA Purification Kit for Blood Version II (Epicentre, USA). *MSTN* variants were determined by means amplification created restriction site method (ACRS). Following thermal profile was applied in PCR: 95ºC/5 min, 30 cycles of 95ºC/45 s, 55ºC/45 s, 72ºC/45 and 72ºC/5 min. The following PCR primers within intron 1 were designed manually: MSTNF 5' – ATTTGATAGCAGAGTCATAAAGGAAAAGTA – 3' MSTNR 5' – CTGCGATCCTGCTTTACCCA – 3'. Underlined nucleotide in primer MSTNF indicates mismatch which creates artificial restriction site. Primers parameters were determined utilizing OligoCalc software (Kibbe 2005). Restriction analysis was carried out by use *In silico* (Bikandi et al. 2004). Amplification reactions were performed in a total volume of 15 µl containing 80–100 ng of DNA, 2xPCR Master Mix (A&A Biotechnology, Poland), 10 pmol of each primer and ddH_2O. Obtained amplicons were digested by 3U of *Rsa*I enzyme (5'GT↓AC3') (*Thermo Scientific, USA*) in 37ºC overnight. Restriction fragments were separated in 3% agarose gels stained with ethidium bromide. Results were visualized and recorded in UV light by use Vilber Lourmat system (France).

Population parameters – genotypes and alleles frequency, gene diversity (expected heterozygosity) and Hardy-Weinberg equilibrium were calculated by means PowerMarker software (Liu and Muse 2010). To compare genotypes frequencies between breeds χ^2 test was applied where: H_0 – lack of differences between breeds, H_1 – differences between breeds are present. Calculations were performed by use Statistica ver. 10 (Statsoft, USA).

RESULTS AND DISCUSSION

PCR method allowed to obtain amplicons with expected length – 132bp. *MSTN* gene variants were determined after *Rsa*I enzyme digestion based on following restriction fragments lengths: *TT* – 132bp, *CT* – 132, 103, 29bp and *CC* – 103, 29bp (Fig.1).

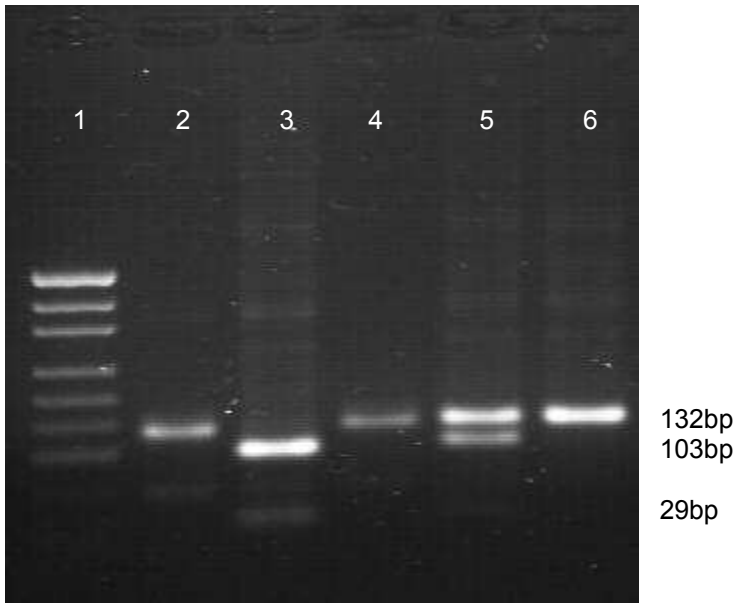

Fig. 1. Electrophoretic separation of restriction fragments in 3% agarose gel. Lane 1 – pUC19/*Msp*I DNA marker (*Thermo Scientific, USA*), lanes 2, 4, 6 – *TT* genotype, lane 3 – *CC* genotype, lane 5 – *CT* genotype
Ryc. 1. Rozdział elektroforetyczny fragmentów restrykcyjnych w 3-procentowym żelu agarozowym. Ścieżka 1 – marker DNA pUC19/*Msp*I (*Thermo Scientific, USA*), ścieżki 2, 4, 6 – genotyp *TT*, ścieżka 3 – genotyp *CC*, ścieżka 5 – genotyp *CT*

Population statistics data calculated for analyzed equine breeds are presented in the Table 1.

Obtained results indicate that the speed variant (*CC* genotype) was present only in 5 individuals belong to Holstein breed, which represents saddle type. It seems that is outcome of this breed formation by use English Thoroughbred stallions as well as directional selection for sport prowess. In other breeds studied only two genotypes appeared – *CT* and *TT*. Highest frequency of heterozygous genotype was observed in Polish Noble Half-breed (0.735). *CT* variant represents mix of speed and stamina and is preferred for fast, middle distance horses. *TT* variant was present with highest frequency in Polish Heavy Draft and Polish Konik horses (0.830 and 0.736 respectively). It is very high correlated with the way of use these horses by work, where physical effort is high and long, but not connected with speed. Highest value of gene diversity was found in Holstein (0.406), however lowest in Polish Heavy Draft horses (0.155). The genotypes distribution was in Hardy-Weinberg equilibrium in all analyzed breeds. The comparison of genotypes frequencies showed statistically significant differences between analyzed equine breeds ($p = 0.0162$ for *CC*, $p = 0.0002$ for *CT* and $p = 0.0037$ for *TT*).

Table 1. Genotypes and alleles frequency with expected heterozygosity (He) calculated for *MSTN* gene polymorphism in analyzed breeds of horses
Tabela 1. Frekwencja genotypów i alleli wraz z oczekiwaną heterozygotycznością (He), obliczone dla genu *MSTN* u badanych ras koni

Utility type / breed Typ użytkowy / rasa	n	Alleles frequency Frekwencja alleli		Genotypes frequency Frekwencja genotypów			He
		C	*T*	*CC*	*CT*	*TT*	
Saddle / Polish Noble Half-breed Wierzchowy / polski koń szlachetny półkrwi	34	0.132	0.868	–	0.735	0.265	0.230
Saddle / Holstein Wierzchowy / holsztyńska	69	0.283	0.717	0.073	0.420	0.507	0.406
Heavy-draught / Polish Heavy Draft Ciężkozaprzęgowy / polski koń zimnokrwisty	53	0.085	0.915	–	0.170	0.830	0.155
General / Polish Konik Wszechstronnie użytkowy / konik polski	53	0.132	0.868		0.264	0.736	0.229

Results mentioned above confirm studies of other authors who stated that horse breeds raised for short distance races, where demonstration of high speed in short time is needed are characterized by higher frequency of *CC* genotype. Quarter Horses are the good example, where *CC* genotype was found with frequency of 0.83, however *TT* – 0.03. *TT* genotype is present very rarely in racehorses, in contrast to horses known from extremely endurance as Egyptian Arabians – 0.90 (Hill et al. 2010). Bower et al. (2012) analyzed g.66493737C/T polymorphism in horses from 22 populations to trace the ancestry of the *C* allele. They showed highest prevalence of *TT* genotype in trotters – French Trotter (0.98) and Standardbred (1.00) which are characterized by good stamina. Moreover results obtained clearly indicated that *C* allele is not restricted to the Thoroughbred and Thoroughbred-derived populations. Authors found that this mutation is not new and seems to appear at variable frequencies which depend on the selection pressures on the population.

Analysis of polymorphism in position g.66493737 was carried out for the first time in horses raised in Poland. Recent studies, concerning five of the most common breeds in Poland were focused on single nucleotide polymorphisms (SNPs) localized in the regulatory part of *MSTN* gene. Two single nucleotide polymorphism (g.66495826T/C and g.66495696T/C) and their haplotypes were analyzed in Arabian, Thoroughbred, Polish Konik, Hucul and Polish Heavy Draft horses. Highly significant differences ($P < 0.001$) were observed between pairs of breeds in frequencies of genotypes. Moreover new haplotype was indentified in Polish Heavy Draft horses (Stefaniuk et al. 2014). Previous analysis showed that *C* alleles in both positions presented higher frequency in heavy than in light breeds and that g.66495826T/C variants are associated with some morphological traits in the Italian Heavy Draft Horse breed (Dall'Olio et al. 2010, 2014).

CONCLUSION

Designed PCR based test – ARMS allowed to determine all of the genotypes in the equine *MSTN* gene. Statistically significant differences were found between genotypes frequencies among four analyzed breeds of horses raised in Poland, which represent different utility types.

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Abstract. Gene encoding myostatin – *MSTN* is a marker for race distance, speed indices and body composition in Thoroughbred racehorses . Because it shows high variability depends on breed and utility type the aim of this study was to: design PCR based test for g.66493737C/T polymorphism analysis in intron 1 of the equine *MSTN* gene; analyze occurrence of g.66493737C/T variants in four breeds of horses, raised in Poland. Investigations were carried out on 209 horses belong to the following breeds: Holstein, Polish Noble Half-breed, Polish Heavy Draft and Polish Konik. For *MSTN* gene analysis amplification created restriction site method (ACRS) was applied. Digestion of *MSTN* gene amplicons (132bp) by *Rsa*I enzyme allowed to discriminate individual genotypes based on following restriction fragments lengths: *TT* – 132bp, *CT* – 132, 103, 29bp and *CC* – 103, 29bp. Obtained results showed that *CC*

genotype (speedy, sprint type) was present only in Holstein breed (0.073), which is known to have English Thoroughbreds ancestors. *CT* genotype (fast, middle-distance type) appeared with highest frequency (0.735) in Polish Noble Half-breed horses. Both of breeds represents saddle type. Last genotype – *TT* (greater stamina) was most common in Polish Heavy Draft (0.830) and Polish Konik (0.736) horses which are classified to heavy-draught and general type respectively. Differences in genotypes frequencies between breeds were statistically significant ($p \leq 0.05$ for *CC*, $p \leq 0.01$ for *TT* and $p \leq 0.001$ for *CT*). Results of this study showed *MSTN* gene variants differentiation depends on utility type and breed of horses raised in Poland.

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